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Effect of Leucine on Enzymes of the Tryptophan–Niacin Metabolic Pathway in Rat Liver and Kidney

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Dietary excess of leucine affects tryptophan–niacin metabolism adversely and has thus been implicated in the etiology of pellagra. To understand the biochemical basis of leucine-induced changes in tryptophan–niacin metabolism the effect of leucine on enzymes of tryptophan–niacin metabolism was investigated. Excess of leucine in the diet had no effect on rat liver 3-hydroxyanthranilate oxygenase and nicotinate phosphoribosyltransferase but significantly decreased the activity of quinolinate phosphoribosyltransferase of rat liver and kidney. The activities of tryptophan oxygenase in liver and picolinate carboxylase in kidney were significantly higher in leucine-fed animals than in the controls. Also, oxidation of [U-^{14}C]tryptophan *in vivo* was higher in leucine-fed animals. Increased picolinate carboxylase and decreased quinolinate phosphoribosyltransferase activities would result in a decrease in NAD formation from dietary tryptophan. Lowered NAD formation from tryptophan particularly when the niacin concentrations in the diet are marginal would result in a state of conditioned niacin deficiency.

Pellagra, though traditionally associated with consumption of maize, also occurs in populations whose staple is jowar (*Sorghum vulgare*). One common feature between maize and jowar is the presence of relatively large amounts of leucine in their proteins as compared with other cereals. It was postulated that excess of leucine in jowar may play a role in the pathogenesis of pellagra (Gopalan & Srikantia, 1960). Though doubts were expressed about the link between a dietary excess of leucine and pellagra (Truswell *et al.*, 1963; Truswell, 1963), exhaustive studies have provided conclusive evidence in support of this hypothesis (Belavady & Gopalan, 1965; Belavady *et al.*, 1967; Madhavan *et al.*, 1968; Gopalan *et al.*, 1969). Leucine feeding has also been shown to alter the urinary excretion of tryptophan–niacin metabolites, particularly quinolinic acid (Belavady *et al.*, 1963; Raghuramulu *et al.*, 1965*a*). Erythrocytes of patients suffering from pellagra (pellagrins) and normal humans supplemented with leucine had lowered capacity to synthesize NAD *in vitro* (Raghuramulu *et al.*, 1965*b*).

To understand the biochemical basis of leucine-induced changes in tryptophan–niacin metabolites, the effects of dietary leucine on the enzymes involved in tryptophan–niacin metabolism were investigated. The enzymes studied were tryptophan oxygenase (EC 1.13.1.12), 3-hydroxyanthranilate oxygenase (EC 1.13.1.6), picolinate carboxylase, quinolinate phosphoribosyltransferase (EC 2.4.2.–) and nicotinate phosphoribosyltransferase (EC 2.4.2.11). Oxidation of tryptophan to CO_2 *in vivo* was also studied. Increased excretion of quinolinic acid in rats on a 9%-casein diet supplemented with leucine but containing no added niacin was reported to be modified by the inclusion of niacin in the diet. Addition of niacin itself caused increased excretion of quinolinic acid (Raghuramulu *et al.*, 1965*a*). In view of these observations, the effect of leucine on the above enzymes in the presence and in the absence of niacin added to the diet was also studied.

Materials and Methods

Chemicals

[*carboxyl*-^{14}C]Nicotinic acid (1.8μCi/μmol) was obtained from Bhabha Atomic Research Centre, Trombay, India. Phosphoribosyl 1-pyrophosphate (magnesium salt), quinolinic acid and ATP were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. DL[*benzene-ring*-U-^{14}C]Tryptophan was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [2,3,7,8-^{14}C]Quinolinic acid (1.77μCi/μmol) was a gift from Dr. Y. Nishizuka. L-Leucine was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and was free from isoleucine.

Diets

A basal diet containing 9% protein derived from crude casein was employed. The composition of the diet was similar to that reported by Raghuramulu *et al.* (1965*a*). The vitamin mixture added to the diet

contained all vitamins except niacin. The niacin content of the basal diet was 0.5mg/100g. When niacin was included in the diet it was added at 1mg/100g of diet. L-Leucine was supplemented at 1.5 or 3% (w/w) at the expense of starch.

Animals

Young albino rats (inbred strain of National Institute of Nutrition), 28-days old, were grouped so that sex, litter and initial body weights were similar.

The animals were fed for a period of 4–5 weeks during which their body weights and food intakes were recorded. At the end of the feeding period equal numbers of animals from each group belonging to the same litter were killed between 00:10h and 00:11h by a blow on the head and the liver and kidneys were removed for the determination of enzyme activities.

Enzyme assays

Tissue (liver or kidney) was homogenized in the cold (2°C) for 3min with 2vol. of 0.14M-KCl in a glass–Teflon homogenizer. The homogenate was centrifuged for 20min at 20000*g* at 2°C. The supernatant solution was treated for 5min with charcoal (15g/100ml of enzyme solution). The charcoal was subsequently removed by centrifugation at 4°C. This preparation was used for the assay of quinolinate phosphoribosyltransferase and nicotinate phosphoribosyltransferase, picolinate carboxylase and 3-hydroxyanthranilate oxygenase. For tryptophan oxygenase, liver was homogenized with 9vol. of 0.14M-KCl in 0.0025M-NaOH, centrifuged for 2min at 600*g* and the supernatant used for the assay of the enzyme. Quinolinate phosphoribosyltransferase was assayed by the microbiological procedure described by Nishizuka & Nakamura (1970), with *Lactobacillus arabinosus* (A.T.C.C. 8014) as the test organism. In one of the experiments, this enzyme was assayed by the radiometric method described by Nishizuka & Nakamura (1970).

Activity of nicotinate phosphoribosyltransferase was assayed as described by Ikeda *et al.* (1965), except that 40μmol of NaF was used in the incubation mixture to minimize 5-phosphoribosyl 1-pyrophosphate breakdown (Ismande, 1964). Picolinate carboxylase was assayed spectrophotometrically by the method of Nishizuka *et al.* (1970). Tryptophan oxygenase and 3-hydroxyanthranilate oxygenase were assayed spectrophotometrically by the methods of Knox (1951) and of Mehler *et al.* (1958) respectively as described by Chiancone (1965). Protein in all enzyme preparations was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Since significant litter variation was observed, statistical analysis was carried out by using analysis of variance and Fisher's *F* test.

Oxidation of tryptophan to CO_2 in vivo

Oxidation of tryptophan *in vivo* via the glutarate pathway was determined in six animals by measuring $^{14}CO_2$ expired after injection of DL-[*benzene-ring*-U-^{14}C]tryptophan by the method of Nishizuka & Hayaishi (1971).

Results

The presence of excess of leucine in the diet did not influence either growth or food intake of rats. Table 1 shows results of the experiments carried out to test the effect of leucine on liver and kidney quinolinate phosphoribosyltransferase in the presence and in the absence of added niacin in the diet. In all three experiments it was observed that 3% (but not 1.5%) leucine in the diet significantly decreased the activity of liver quinolinate phosphoribosyltransferase. In the first experiment the liver quinolinate phosphoribosyltransferase was studied by feeding leucine in two different amounts. The results of Expt. 2, where the radiometric method of quinolinate phosphoribosyltransferase assay was used, confirmed the observations made by the microbiological method in the other two experiments. Although kidney quinolinate phosphoribosyltransferase activity was inhibited significantly by 3% leucine when the diet contained no added niacin, inhibition was not observed when the diet contained added niacin. Addition of niacin *per se* to the diet at 1mg/100g also did not affect the quinolinate phosphoribosyltransferase activity. When quinolinate phosphoribosyltransferase activity was measured in control and leucine-fed animals at different enzyme concentrations (corresponding to 3–8mg of protein) the activity in the leucine-fed animal was observed to be low at all enzyme concentrations.

The results of Expt. 1 (Table 2) show that when leucine was included in the diet in the presence of added niacin the activity of liver tryptophan oxygenase was significantly increased whereas that of 3-hydroxyanthranilate oxygenase was unaffected. No measurable activity of picolinate carboxylase was detected in livers of either the control or the leucine-fed animals. In the kidney, however, measurable activity of this enzyme was present (Table 2). These observations on the activity of picolinate carboxylase in kidney and liver in the control animals are in line with that of Lan & Gholson (1965). The results show that the activity of this enzyme increased with increasing content of leucine in the diet, and the activity in rats fed 3% leucine was significantly higher compared with that in control or 1.5%-leucine-fed animals.

In Expt. 2 (Table 2) the effect of leucine was studied in the presence and in the absence of added niacin in

Table 1. *Effect of leucine on rat liver and kidney quinolinate phosphoribosyltransferase activity*

In Expts. 1 and 3 activity is expressed as nmol of niacin ribonucleotide formed/h per mg of protein. The complete incubation mixture contained 100nmol of quinolinic acid, 400nmol of 5-phosphoribosyl 1-pyrophosphate, 50μmol of Tris–acetate buffer (pH7), 1μmol of $MgCl_2$ and approx. 6mg of the supernatant protein in a total volume of 1ml. Incubation was carried out for 45min at 37°C. The niacin ribonucleotide formed was hydrolysed to free niacin by heating for 30min at 100°C in 0.1M-NaOH. This was then neutralized to pH6.8 and the niacin was estimated by microbiological assay by using *L. arabinosus* (A.T.C.C. 8014). In Expt. 2 the radiometric method of assay for quinolinate phosphoribosyltransferase was used and the activity was expressed as nmol of $^{14}CO_2$ produced/h per mg of protein. The conditions of incubation were exactly the same except that [2,3,7,8-^{14}C]quinolinic acid was used instead of unlabelled quinolinic acid. Incubation was carried out in Warburg flasks with 0.1ml of Hyamine hydroxide in small cups placed in the central well. The $^{14}CO_2$ trapped by Hyamine hydroxide was measured in toluene scintillator [toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene] by using a Packard Tri-Carb liquid-scintillation spectrometer. Numbers of animals are given in parentheses. N.S., not significant.

	Treatment	Enzyme activity: Liver	Enzyme activity: Kidney
Expt. 1	A. Control+niacin	1.39 (5)	—
	B. Control+niacin+ 1.5% leucine	1.23 (4)	—
	C. Control+niacin+ 3% leucine	0.94 (5)	—
	Pooled S.D. ...	0.16	
Significance of difference	...A and B, N.S.	A and C, $P<0.01$	B and C, $P<0.05$
Expt. 2	A. Control+niacin	1.93	—
	B. Control+niacin+ 3% leucine	1.31	—
	Pooled S.D. ...	0.34 (5)	
Significance of difference	...	A and B, $P<0.05$	
Expt. 3	A. Control	1.10	1.86
	B. Control+3% leucine	0.80	1.42
	Pooled S.D. ...	0.15 (8)	0.31(7)
Significance of difference	...	A and B, $P<0.01$	A and B, $P<0.05$
	A. Control+niacin	1.36	1.92
	B. Control+niacin+ 3% leucine	0.97	2.00
	Pooled S.D. ...	0.25 (9)	0.30 (8)
Significance of difference	...	A and B, $P<0.025$	A and B, N.S.

the diet. In this experiment only the liver tryptophan oxygenase and kidney picolinate carboxylase activities were determined. The results show that the activities of both these enzymes were markedly increased by incorporation of leucine in the diet whether or not the diet contained added niacin. In the presence of niacin added to the basal diet the activity of tryptophan oxygenase tended to be lower and that of kidney picolinate carboxylase showed a significant increase. The activity of nicotinate phosphoribosyltransferase was not altered by feeding leucine at different concentrations of niacin in the diet. The activity was around 2.0nmol of product formed/h per mg of protein in all the groups.

Fig. 1 shows $^{14}CO_2$ expired by the control and leucine-fed animals at different time-intervals. Expired $^{14}CO_2$ expressed as a percentage of injected dose was higher in leucine-fed animals than in controls.

Discussion

The effect of leucine on enzymes of tryptophan metabolism is summarized in Scheme 1. The importance of changes in these enzymes in the context of

Table 2. *Effect of leucine on tryptophan oxygenase and 3-hydroxyanthranilate oxygenase activities of liver and picolinate carboxylase of kidney*

Activity of tryptophan oxygenase determined in homogenate is expressed as μmol of kynurenine formed/h per 100mg of N. 3-Hydroxyanthranilate oxygenase activity is expressed as ΔE_{360}/min per mg of N, where ΔE_{360} is the difference between E_{360} before and 1 min after addition of the substrate. The incubation mixture for picolinate carboxylase contained 50μmol of potassium phosphate buffer, pH7.5, and aqueous extract corresponding to 10mg of acetone-dried powder (Colowick & Kaplan, 1955) in a total volume of 2.8ml. The reaction was started by addition of 70nmol of 3-hydroxyanthranilic acid to the test cuvette. At 2min when the formation of α-amino-β-carboxymuconic ε-semialdehyde was complete approx. 3mg (in 0.1ml) of the tissue supernatant protein was added and the rate of disappearance of α-amino-β-carboxymuconic ε-semialdehyde was recorded at 360nm against a blank incubation mixture that contained all ingredients except 3-hydroxyanthranilic acid. Activity was expressed as nmol of substrate disappeared/min per mg of protein. Numbers of animals are given in parentheses. N.S., not significant.

		Liver		Kidney
	Treatment	Tryptophan oxygenase activity	3-Hydroxyanthranilate oxygenase activity	Picolinate carboxylase activity
Expt. 1	A. Control+niacin	3.40 (7)	7.94 (10)	4.04 (7)
	B. Control+niacin+ 1.5% leucine	—	—	4.69 (7)
	C. Control+niacin+ 3% leucine	4.56 (8)	6.89 (11)	5.54 (7)
	Pooled S.D. ...	0.84	1.34	0.93
	Significance of difference...	A and C, $P<0.05$	A and C, N.S.	A and B, N.S. B and C, $P<0.05$ A and C, $P<0.01$
Expt. 2	A. Control	3.03	—	4.51
	B. Control+3% leucine	4.18	—	5.15
	Pooled S.D. ...	0.51 (8)		0.49 (9)
	Significance of difference...	A and B, $P<0.01$		A and B, $P<0.05$
	C. Control+niacin	1.76	—	5.33
	D. Control+niacin+ 3% leucine	3.27	—	6.53
	Pooled S.D. ...	1.14 (8)		0.92 (7)
	Significance of difference...	C and D, $P<0.05$		C and D, $P<0.05$
	Effect of niacin *per se*, i.e. significance of difference between A and C ...	$P<0.10$		$P<0.001$

tryptophan utilization for NAD synthesis can be judged by considering total activities of these enzymes in liver and kidney of the control and leucine-fed animals, which shows the rate-limiting enzyme in the formation of NAD from tryptophan is quinolinate phosphoribosyltransferase. Although increased tryptophan oxygenase activity can supply increased amounts of substrates for picolinate carboxylase and for conversion into quinolinic acid, it may not affect NAD formation since the latter is limited by the quinolinate phosphoribosyltransferase activity. Since picolinate carboxylase activity is several times higher than that of tryptophan oxygenase any change in the activity of the former may have significant influence on the amount of the intermediate converted into quinolinic acid. It is therefore evident that the enzymes, whose altered activities would affect NAD formation from tryptophan, are primarily picolinate carboxylase and quinolinate phosphoribosyltransferase. Picolinate carboxylase and quinolinate phosphoribosyltransferase are the two important enzymes of the glutarate (Ichiyama *et al.*, 1965) and NAD (Nizhizuka & Hayaishi, 1963*a*,*b*) pathways respectively of tryptophan metabolism and are known to control the amount of NAD formed from tryptophan. An inverse relationship between these two enzymes has been demonstrated in the present study. In leucine-fed animals quinolinate phosphoribosyltransferase activity decreased and that of picolinate carboxylase increased relative to their values in the

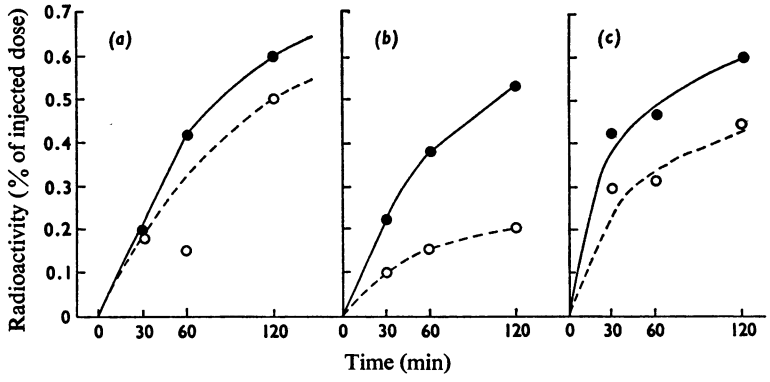
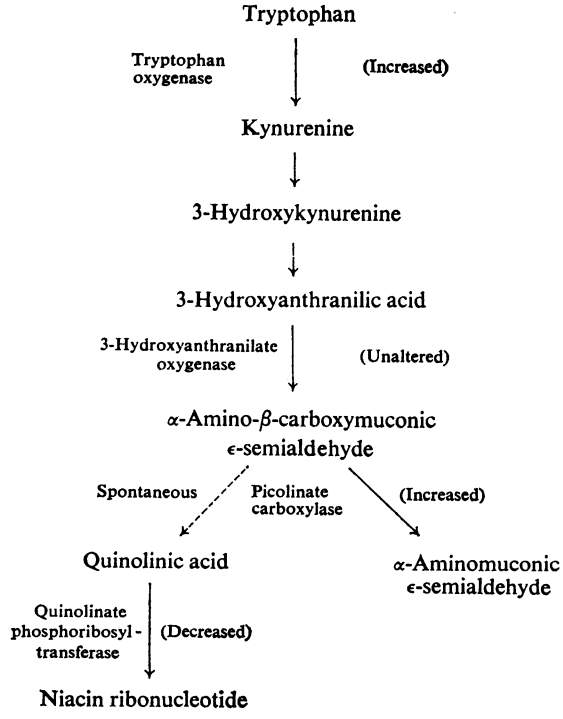


Fig. 1. *Effect of leucine on oxidation of tryptophan in vivo*

An accurately measured amount of radioactive tryptophan solution in 0.85% NaCl (4μCi/ml) containing about 1μCi was injected into the peritoneal cavity of rats and expired $^{14}CO_2$ was collected over the next 2h. $^{14}CO_2$ expired during the $\frac{1}{2}$, 1 and 2h intervals was trapped in 1M-NaOH and precipitated as $Ba^{14}CO_3$. The $Ba^{14}CO_3$ precipitate was washed several times with water (until free of NaOH) and subsequently with acetone and dried overnight at 160°C. To thixotropic gel powder (Cab-O-Sil) in a 25ml counting vial, a weighed amount of $Ba^{14}CO_3$ precipitate and 12ml of scintillator [15g of naphthalene, 0.7g of 2,5-diphenyloxazole and 0.03g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 100ml of distilled dioxane] were added and vigorously shaken to obtain a homogenous suspension. This was then counted in a Packard Tri-Carb liquid-scintillation spectrometer. From the specific radioactivity of $Ba^{14}CO_3$ and total yield of $Ba^{14}CO_3$ at different time-intervals the total expired $^{14}CO_2$ was calculated and expressed as a percentage of the injected dose. ○, Control; ●, control+3% leucine. (*a*), (*b*) and (*c*) represent three pairs of control and experimental rats.



Scheme 1. *Effect of leucine on enzymes of tryptophan metabolism*

The effect of leucine at each stage is shown in parentheses.

controls. A decreased activity of quinolinate phosphoribosyltransferase may also partly explain the increased quinolinic acid excretion observed in the presence of excess of leucine in the diet (Raghuramulu *et al.*, 1965*a*; Belavady *et al.*, 1963). The fact that leucine in the presence of added niacin decreased the enzyme activity without affecting quinolinic acid excretion (Raghuramulu *et al.*, 1965*a*) suggests other mechanisms contributing to increased quinolinic acid excretion.

Our observation of the effect of leucine on the important enzymes of tryptophan metabolism can offer a biochemical explanation for the role of leucine in the etiology of pellagra. Increased picolinate carboxylase and decreased quinolinate phosphoribosyltransferase activities would result in a decrease in NAD formation from dietary tryptophan. Lowered NAD formation from tryptophan, particularly when the niacin concentrations are marginal, would result in a state of conditioned niacin deficiency. Experimental niacin deficiency (pellagra) has been induced in humans on intakes of tryptophan and niacin amounting to 4.0 niacin equivalents (niacin equivalent = dietary niacin plus 1/60 of dietary tryptophan) or less/kcal, but not on intakes of 4.4 niacin equivalents/kcal (Goldsmith, 1958). In the presence of adequate niacin, however, although the enzyme changes are present they may not affect the NAD pool since adequate NAD may be formed from the dietary niacin. The activity of nicotinate phosphoribosyltransferase is not altered by a dietary excess of leucine.

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